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The Effect of Actinomycin D on Primary Embryonic Induction[1]

by

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With 4 text figures.

Actinomycin D, a chromopeptide discovered by Vining and Waksman (1954) has proved to be a powerful analytical tool in studies of RNA synthesis. The observations of Reich *et al.* (1962) and Hurwitz *et al.* (1962) indicate that actinomycin D inhibits the synthesis of the entire complement RNA (nuclear, ribosomal and transfer). It does not, however, interfere with the turnover of terminal nucleotides and some recent studies have shown the existence of actinomycin-resistant RNA-synthesis in animal cells (Paul and Struthers, 1963).

Since RNA synthesis is intimately involved in cellular differentiation, actinomycin D has been employed by several workers in the field. There is some preliminary evidence in favour of actinomycin being capable of inhibiting neural differentiation in amphibian embryos (Brachet and Denis, 1962, Denis, 1963). If applied to sea urchin eggs at early developmental stages, actinomycin interferes with uracil uptake and morphogenesis (Gross and Cousineau, 1963). In this note we report upon some experiences gained in our laboratory with the use of actinomycin D as an inhibitor of primary embryonic induction.

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In the pilot experiments, the lines of BRACHET and DENIS (1962) were followed employing another species, *Triturus vulgaris*. Either whole, uncoated young gastrulae or " sandwich "-type explants were treated in an actinomycin solution, and subsequently their morphogenesis was followed microscopically. Whole gastrulae were cultivated for 15 days in concentrations ranging from 1 to 20 µg/ml of actinomycin D, and normal development was obtained in most cases. Only 9 malformed larvae were obtained in the series of 75 embryos and these were randomly distributed between the batches kept in different concentrations and thus were hardly due to the drug applied.

Subsequently, the gastrula ectoderm was exposed to actinomycin D in experiments where the explantation technique was employed. 6-12 hours treatment of the explants in concentrations of 0.5 to 2.5 µg/ml did not affect the morphological differentiation. Microscopical analysis of 15 cases treated in this way, yielded a normal spectrum of induced organs.

The obvious discrepancy between these observations and the results of BRACHET and DENIS (1962) may find their explanation in differences in the penetration of the drug into the ectodermal cells of the different species used. When explants of *Triturus vulgaris* were tested for their capacity to incorporate H^3-uridine, the uptake proved to be very poor and completely independent of the presence of actinomycin D employed at a concentration of 10 µg/ml. This poor penetration may furthermore, explain some of our earlier results. In a previous report we have stated that 5-fluorouracil effectively inhibits neural induction (TOIVONEN *et al.*, 1961), but recent, identical experiments with 5-fluorouridine have yielded negative results (unpublished). Hence, in the recent studies disaggregated cells have been exposed to actinomycin and/or uridine to obtain a better incorporation, and, possible a better effect.

METHOD

The competent ectoderm of young *Triturus vulgaris* embryos was employed to make " sandwich "-type explants with guinea pig bone marrow as the inducer (TOIVONEN, 1954). After 24 hours when the induction was completed (TOIVONEN, 1958), the inductor tissue was removed and the ectodermal pieces were disaggregated in Mg-free EDTA at pH 7.8 (Jones and Elsdale, 1963). The disaggregated cell suspension was thereafter transferred to normal culture medium containing actinomycin D at various concentrations.

For morphological studies the reaggregated cells were transferred after 6 hours exposure to actinomycin D into normal culture medium, subsequently cultivated for 12 days at +18°, sectioned and analyzed microscopically.

In the incorporation experiments, the cell suspension after disaggregation was transferred to a medium containing uridine H^3 (0.2 μC/ml) with or without actinomycin D (10 μg/ml). In the final experiments the time of incubation was 12 hours, after which the acid-insoluble RNA was determined using a liquid scintillation counter (I.D.L.). The ectodermal cells were disrupted in 20 per cent NaCl before treatment with 0.5 M perchloric acid. This was followed by absolute alcohol and hyamine hydroxide. For each determination 5 ectodermal explants were pooled.

Control explants were made without any inducing tissue, and subsequently treated identically with the sandwiches induced by bone marrow.

RESULTS

Morphology. The first steps of development after transferring the cells to normal medium were similar in all actinomycin experiments and controls. The cells reaggregated in 3-4 hours forming a dense, ball-like cluster. In the control series and in the specimens treated by low concentrations of actinomycin D (0.005-0.05 μg/ml) this cluster showed differentiation in a few days, and when analyzed after 12 days of cultivation, the spectrum of differentiated tissues was identical to the known inductive product of bone marrow. (Fig. 1).

When treated with actinomycin in concentrations of 2.5 μg/ml to 10 μg/ml the development was markedly different. After 24-36 hours the originally coherent reaggregated cell cluster disaggregated again and formed an irregular, pancakelike cell mass (Fig. 2). During the subsequent 10 days of cultivation no differentiation of these cells could be obtained, although they

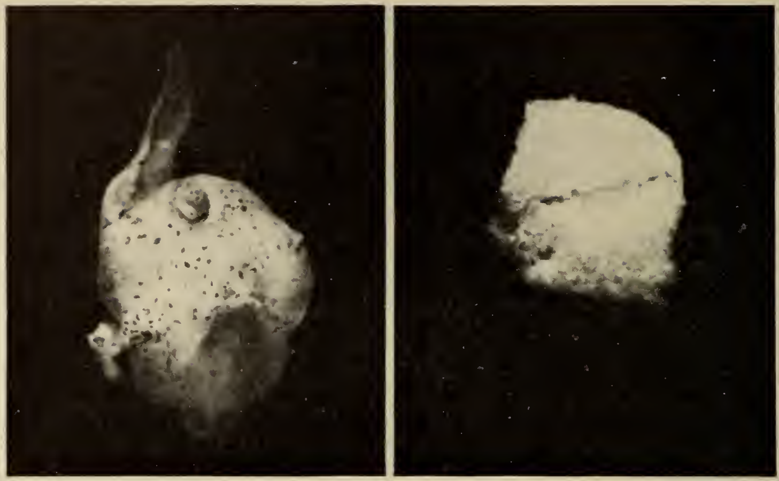

Fig. 1 and 2.

Two examples of actinomycin-treated ectodermal explants. Subsequent to induction by bone marrow the ectoderms were disaggregated and treated with actinomycin D in concentrations of 0.5 μg/ml (Fig. 1), and 2.5 μg/ml resp. (Fig. 2). Differentiation of spinocaudal structures was noted in the explant treated with the low concentration whereas the higher concentration completely inhibited this morphogenesis. Samples were photographed after 12 days of cultivation.

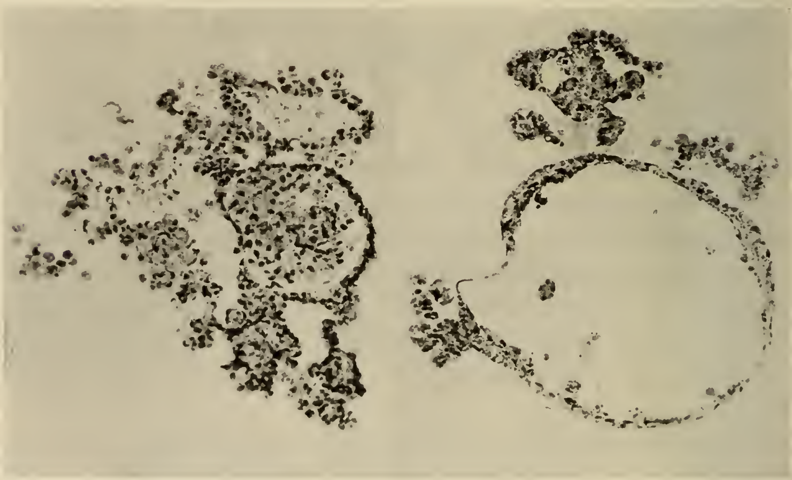

Fig. 3 and 4.

Two examples of ectodermal reaggregates previously treated with actinomycin D at a concentration of 1 μg/ml. The microscopical sections show some epidermal differentiation but no other structures. Fixed after 12 days of cultivation.

were obviously viable. (This was proved by neutral red incorporation as well as by incorporation experiments with leucine-H^3).

Specimens treated with actinomycin in a concentration 1 μg/ml showed an intermediate development, where most of the cells become disaggregated and remained undifferentiated around a small denser nodule showing epidermis-like differentiation but no other structures (Figures 3 and 4). The results of the microscopical analysis are given in Table 1.

TABLE 1.

Differentiation of actinomycin D-treated ectodermal explants.

Concentration	Number of explants	Survival	Normal differentiation	No differentiation	Epidermal differentiation
0.005 μg/ml	4	4/4	4/4	0	4/4
0.05 "	5	5/5	5/5	0	5/5
0.5 "	12	11/12	10/11	1	10/11
1.0 "	7	5/7	0	1/5	4/5 *
2.5 "	6	The cells show incorporation of neutral red and leucine-H^3. Morphologically normal nuclei are seen as well as yolk granules after 12 days of cultivation. No differentiation can be noted.			
10.0 "	6				

* Epidermal-like differentiation but no other formations.

Uridine incorporation. The results of the final incorporation experiments are given in Table 2.

TABLE 2.

Incorporation of uridine H^3 into disaggregated ectodermal cells from sandwiches induced by guinea pig bone marrow and the effect of actinomycin D on this incorporation.

	Actinomycin D	Counts per min. *	
		Exp. 1	Exp. 2
Induced	none	431	384
Induced	10 μg/ml	213	120
Non-induced	none	414	204

* Per five sandwiches.

COMMENT

The results presented above indicate that when brought into the disaggregated condition, the induced ectodermal cells are sensitive to actinomycin D, which, in concentrations from 1 μg/ml seems to prevent cytodifferentiation. The primary effect morphologically observable is a secondary disaggregation of the previously dense reaggregate of the determined cells. The same concentrations of actinomycin do not effect the differentiation of whole gastrulae or explants prepared from the competent ectoderm. This difference may be due to the poor penetration of the drug into the coated cells as demonstrated in parallel experiments with labelled uridine. In the disaggregated form the incorporation is better, and here actinomycin D seems to interfere with the uptake of H^3-labelled uridine.

ZUSAMMENFASSUNG

Die Einwirkung von Aktinomycin D auf die Differenzierung der Molchkeime *(Triturus vulgaris)* wurde untersucht.

Nach Disaggregation der mit Knochenmarkgewebe induzierten Ektodermexplantate wurde die Differenzierung der determinierten Zellen mit Aktinomycin (Konz. 1 μg/ml und mehr) verhindert. Niedrigere Konzentrationen waren wirkungslos.

Die Ganzkeime und Ektodermexplantate differenzierten sich normal auch nach Behandlung mit höheren Konzentrationen (20 μg/ml bzw. 2.5 μg/ml). Der Widerspruch dieser Resultate gegenüber denen von Brachet und Denis wird besprochen.

RÉSUMÉ

L'action de l'actinomycine D sur la différentiation de l'embryon du Triton (Triturus vulgaris) a été étudiée.

Des explantats d'épiblaste, soumis à l'action inductrice de moelle osseuse, ont été désagrégés. La différentiation des cellules déterminées a ensuite été empêchée par l'actinomycine (concentration 1 μg/ml et plus). Des concentrations plus faibles n'ont eu aucun effet.

Les embryons entiers et les explantats d'ectoderme se différentient normalement même lorsqu'on les soumet à des concentrations supérieures (20 μg/ml resp. 2,5 μg/ml). Les contradictions entre ces résultats et ceux de Brachet et Denis sont discutées.

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